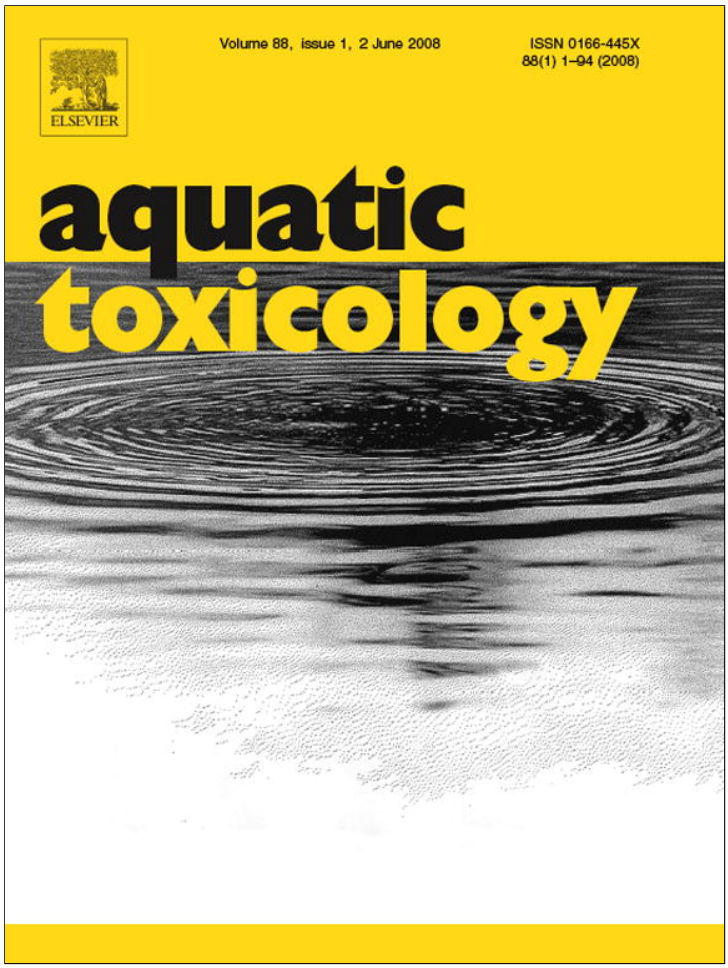

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Relationship of plasma sex steroid concentrations in female fathead minnows to reproductive success and population status

Gerald T. Ankley[a,*], David H. Miller[b], Kathleen M. Jensen[a], Daniel L. Villeneuve[a], Dalma Martinović[a]

[a] US Environmental Protection Agency, Mid-Continent Ecology Division, 6201 Congdon Boulevard, Duluth, MN 55804 USA
[b] US Environmental Protection Agency, Mid-Continent Ecology Division, 9311 Groh Road, Grosse Ile, MI 48138 USA

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ABSTRACT

Concentration and/or production of sex steroids such as 17β-estradiol (E2) and testosterone (T) in fish have commonly been measured in field studies concerned with endocrine-active chemicals. There is a reasonable mechanistic basis for using E2 or T as biomarkers, as chemicals can alter steroid production through both direct and indirect effects on the hypothalamic-pituitary-gonadal (HPG) axis. There is uncertainty, however, as to what changes in steroid status may mean relative to apical endpoints, such as reproduction, that directly affect population status. In this study, we analyzed data from fathead minnow (*Pimephales promelas*) reproduction studies in which decreases in fecundity were associated with depressed steroid production as a result of chemical exposure. Although the chemicals acted on the HPG axis through different mechanisms, reproductive effects appeared to be expressed through a common pathway, depression of vitellogenin production in females. Plasma concentrations of E2 or T in the females were significantly, positively correlated with fecundity. Linear regression models describing the relationship between E2 or T concentrations and relative fecundity were linked to a population model to predict population trajectories of fathead minnows exposed to chemicals that inhibit steroid production. For example, a population existing at carrying capacity and exposed to a chemical stressor(s) that causes a 50% decrease in E2 production was predicted to exhibit a 92% decrease in population size over a 5-year period. Results of our analysis illustrate a conceptual framework whereby a commonly measured biomarker, sex steroid status, could be linked to individual- and population-level effects in fish.

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1. Introduction

New genomic and bioinformatic tools offer substantial promise for discovery of biomarkers indicative of chemical mode/mechanism of action (MOA; Ankley et al., 2006). Just knowing MOA can reduce some uncertainties in ecological risk assessments for chemicals providing, for example, a basis for extrapolating effects across species (SETAC, 2006). However, for biomarker data to be used for effects assessments, it is necessary to understand relationships between molecular/biochemical responses and apical (whole organism) outcomes reflective of population-level effects (Forbes et al., 2006). Such understanding is lacking for most biomarkers; although they can be altered by exposure to a chemical(s) with specific MOA, the responses may have no clear relationship to adverse effects in the organism. For example, a number of classes of chemicals increase expression and activity of different cytochrome P450 (CYP) enzymes in animals, but CYP induction is difficult to link to actual toxicity of the chemicals (Forbes et al., 2006; Oris and Roberts, 2007). Hence, biomarkers suitable for effects assessments need to reflect not only chemical MOA, but expression of resultant impacts through toxicity pathways of concern.

Many different molecular and biochemical endpoints have been used as biomarkers in ecological studies (for review see SETAC, 1992). A response commonly evaluated in field studies with fish – particularly those concerned with potential effects of endocrine-active chemicals – is sex steroid concentration or production (e.g., McMaster et al., 1991; Van Der Kraak et al., 1992; Goodbred et al., 1997; Dubé and MacLatchy, 2000; Karels et al., 2001; Noaksson et al., 2001; Sepúlveda et al., 2001; Lavado et al., 2004; Oakes et al., 2005; Fentress et al., 2006). From a mechanistic perspective, steroids are reasonable biomarkers. Changes in steroid titers can reflect both direct (inhibition of enzymes involved in biosynthesis or degradation) and indirect (feedback inhibition or stimulation) effects of contaminants on the hypothalamic-pituitary-gonadal (HPG) axis. Correspondingly, many of the field studies cited above have documented alterations (usually decreases) in plasma

* Corresponding author. Tel.: +1 218 529 5147; fax: +1 218 529 5003.
E-mail address: ankley.gerald@epa.gov (G.T. Ankley).

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concentrations or production of 17β-estradiol (E2), testosterone (T), and/or 11-ketotestosterone (KT). Although these types of observations suggest the presence of chemicals that affect the HPG axis, it is unknown what the changes may mean in terms of reproductive fitness.

Miller et al. (2007) recently described how a biomarker, plasma vitellogenin (VTG) concentrations in female fish, could be used to predict egg production and, via modeling, forecast fathead minnow (*Pimephales promelas*) population status. Vitellogenin is a lipoprotein produced in the liver of oviparous female vertebrates through activation of the estrogen receptor by E2. Following secretion to the blood, VTG is deposited in the ovary where it is incorporated into developing oocytes. Miller et al. (2007) analyzed an integrated dataset comprised of 21-d fathead minnow reproduction studies with five chemicals that affect the HPG axis through different mechanisms, but all of which decrease VTG and fecundity. Not surprisingly (given the important role of VTG in egg maturation), there was a strong linear relationship between the two variables ($r^2 = 0.88$) which then could be used as a basis for predicting population-level effects. Four of the five test chemicals evaluated by Miller et al. (2007) likely reduced VTG concentrations through a common mode of action, inhibition of steroid synthesis. The pharmaceutical fadrozole is a selective inhibitor of CYP19 (aromatase), which converts T to E2; as a consequence, female fish exposed to fadrozole exhibit decreased E2, but not T (Ankley et al., 2002). The conazole prochloraz likely decreases steroid synthesis in the fathead minnow both through inhibition of CYP19 and CYP17 lyase, an enzyme "up stream" of T, so plasma concentrations of both T and E2 can be decreased by exposure to the fungicide (Ankley et al., 2005). Finally, two synthetic androgen receptor agonists, 17α- and 17β-trenbolone, appear to decrease steroid production through feedback inhibition in the HPG axis, manifested as decreased plasma concentrations of both T and E2 in fathead minnows (Ankley et al., 2003; Jensen et al., 2006).

The purpose of the present study was to expand on the analysis of Miller et al. (2007) by exploring relationships between plasma steroid (E2, T) concentrations and fecundity in the fathead minnow in the context of population-level responses. We feel that the results of the present study illustrate the conceptual basis for (1) better understanding possible population consequences of altered steroid production, and (2) developing a quantitative linkage between steroid production and fecundity to support systems modeling of the fish HPG axis (Breen et al., 2007; Villeneuve et al., 2007).

2. Materials and methods

Egg production and female VTG, T, and E2 data were from 21-d reproduction tests done at our lab over the past 6 years (Ankley et al., 2001). The experiments were conducted with sexually mature (4–6 month old) fathead minnows from an on-site culture unit. Following a 14–21-d acclimation period, the fish were exposed in a continual-flow (ca. 45 mL/min) system that delivered test chemicals dissolved in Lake Superior water without use of carrier solvents. Exposures were conducted at $25 \pm 1\,^{\circ}C$ under a 16:8 (L:D) photoperiod. The animals were fed brine shrimp to satiation twice daily. Nominal test chemical concentrations, shown in Table 1, were confirmed analytically at least weekly in the exposure tanks. Egg production by the fish was assessed daily, and at conclusion of the 21-d test the animals were anesthetized and blood sampled from the caudal vein/artery using micro-hematocrit tubules. Plasma concentrations of VTG and steroids were measured using an enzyme-linked immunosorbent assay and radioimmunoassays, respectively (Jensen et al., 2001). There was no treatment-related mortality during the experiments. Interested readers are encouraged to consult the papers noted in Table 1 for further chemical and biological details about the reproduction studies.

For our analysis, fecundity was calculated as eggs/female/d based on the cumulative number of eggs from a given treatment group. Concentrations of VTG, T, and E2 are expressed as the median value for all female fathead minnows from a treatment. The experiments used one of two different spawning designs: paired (one female and one male, with six replicates per treatment) or group

Table 1
Fecundity and female plasma vitellogenin (VTG), estradiol (E2), and testosterone (T) concentrations in fathead minnow (*Pimephales promelas*) exposed to four different test chemicals

Chemical	Exposure concentration	Plasma VTG (mg/mL)[a]	Plasma E2 (ng/mL)[a]	Plasma T (ng/mL)[a]	Fecundity (eggs/female/d)[b]
17β-Trenbolone[c]	Control	25.8	4.78	3.82	12.3
	0.005 μg/L	23.6	4.11	4.45	11.7
	0.05 μg/L	9.9	1.82	2.06	5.4
	0.5 μg/L	0.0	0.37	1.08	0.0
	5 μg/L	0.0	0.78	1.90	0.0
	50 μg/L	1.3	1.42	2.94	0.0
17α-Trenbolone[d]	Control	17.9	3.09	5.17	25.8
	0.003 μg/L	13.4	5.23	4.49	20.1
	0.01 μg/L	15.0	4.18	2.00	11.5
	0.03 μg/L	5.6	1.43	0.47	8.8
	0.1 μg/L	1.1	0.73	0.21	0.0
Prochloraz[e]	Control	18.7	4.64	3.86	32.4
	0.03 mg/L	11.6	3.01	4.80	22.2
	0.1 mg/L	7.4	3.35	4.80	8.3
	0.3 mg/L	1.8	0.79	2.06	0.0
Fadrozole[f]	Control	15.1	4.43	DNU[g]	20.5
	2 μg/L	7.6	2.91	DNU	8.9
	10 μg/L	1.0	1.32	DNU	0.0
	50 μg/L	0.0	0.0	DNU	0.0

[a] Plasma VTG, E2, and T were calculated as the median value for all females from any given treatment group.
[b] Fecundity (eggs/female/d) was calculated by dividing final cumulative egg production by number of days of exposure.
[c] Ankley et al. (2003).
[d] Jensen et al. (2006).
[e] Ankley et al. (2005).
[f] Ankley et al. (2002).
[g] DNU: data not used.

(four females and two males, with three replicates per treatment). Regression models and/or correlation analysis were used to assess relationships between T and E2 (dependent variable), T or E2 and VTG (dependent variable), and between T or E2 and fecundity (dependent variable). To normalize for slight differences in baseline conditions (e.g., fish age) across experiments, fecundity, VTG, T, and E2 data used in the regression analyses were expressed relative to control measurements for a given experiment (chemical). Data for the various E2 comparisons came from the studies with prochloraz, 17α- and β-trenbolone, and fadrozole. For the T comparisons, data for fadrozole were not used because it does not inhibit T synthesis (Ankley et al., 2002).

To translate observed fecundity/steroid relationships into potential population-level effects, we used the basic model described by Miller and Ankley (2004) as modified by Miller et al. (2007). Simple linear regression models describing relationships between steroid concentrations and fecundity were used in conjunction with the population model to provide trajectories for fathead minnow populations. Given a larger dataset, the relationships between steroid concentrations and fecundity might be more robustly described by nonlinear rather than linear regression models. However, we felt the amount of data available for this analysis was too limited to reliably and quantitatively evaluate a "best fit" model. Instead, the primary goal of our analysis was to illustrate the conceptual basis for linking altered plasma steroid concentrations in fish to fecundity and, ultimately, population status.

The population model used for this study is a density-dependent logistic matrix model constructed using a Leslie projection matrix (Leslie, 1945) in combination with the logistic equation (Miller and Ankley, 2004; Miller et al., 2007):

$$\boldsymbol{n}_{t+1} = \exp\left(\frac{-rP_t}{K}\right)\boldsymbol{M} * \boldsymbol{n}_t \quad (1)$$

In Eq. (1), $\boldsymbol{n}_{t+1}$ is the vector of population age structure at time $t+1$, $\boldsymbol{n}_t$ is the vector of population age structure at time t, r is the intrinsic rate of increase, P_t is the population size at time t, K is carrying capacity, and $\boldsymbol{M}^*$ is the Leslie matrix containing vital rates (survivorship and fertility) that have been adjusted to include an age-specific percent reduction in fecundity and/or survival rates over the time step t resulting from an exposure. In application of Eq. (1), the effect of depressed plasma concentrations of T or E2 in females on population size can be investigated, given that a predictive relationship between these plasma sex steroids and fecundity can be utilized in formulation of $\boldsymbol{M}^*$ to adjust age-specific values of fecundity rates over the time step t, resulting from chemical exposure. The model of Eq. (1) thus provides a calculation through which toxicants affect the Leslie matrix elements related to baseline reproduction values. In addition, a density-dependent factor affects all the matrix elements related to reproduction and survival by reducing them as the population increases. In applying the logistic matrix model of Eq. (1) in this manner, no additional parameters are required beyond what is found in a combined life and fecundity table, an estimate of carrying capacity, and an estimate of plasma sex steroid levels in female fish of the study population.

Using Eq. (1) in connection with a predictive relationship between plasma sex steroid concentrations and fecundity, we investigated population trajectories for a fathead minnow population existing at carrying capacity and subsequently exposed to chemical stressors that reduce E2 or T by a fixed percentage from an unexposed condition. To provide an indication of relative impact, the output of the model was expressed independent of carrying capacity by plotting population size proportional to carrying capacity at each time step of the model. Output from the model can also be evaluated on the basis of absolute numbers, whereby impacts corresponding to effects on a biomarker of interest can be examined for a given population existing at a specified location within a body of water (Miller et al., 2007).

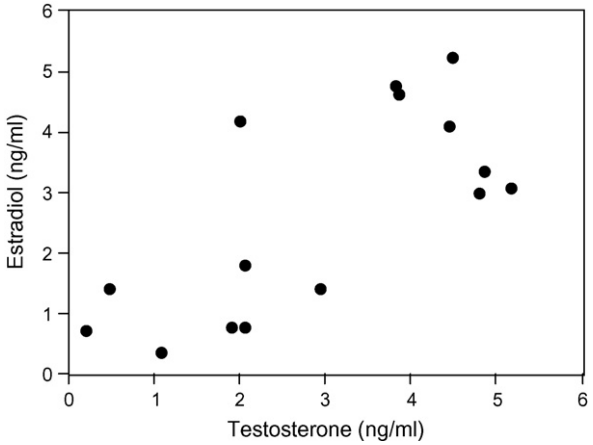

Fig. 1. Relationship between plasma concentrations of estradiol and testosterone (ng/mL) in female fathead minnows exposed to prochloraz, 17α- or 17β-trenbolone for 21 d.

3. Results

Testosterone is the precursor for E2, so the positive correlation ($r = 0.72$) between concentrations of the two steroids observed in

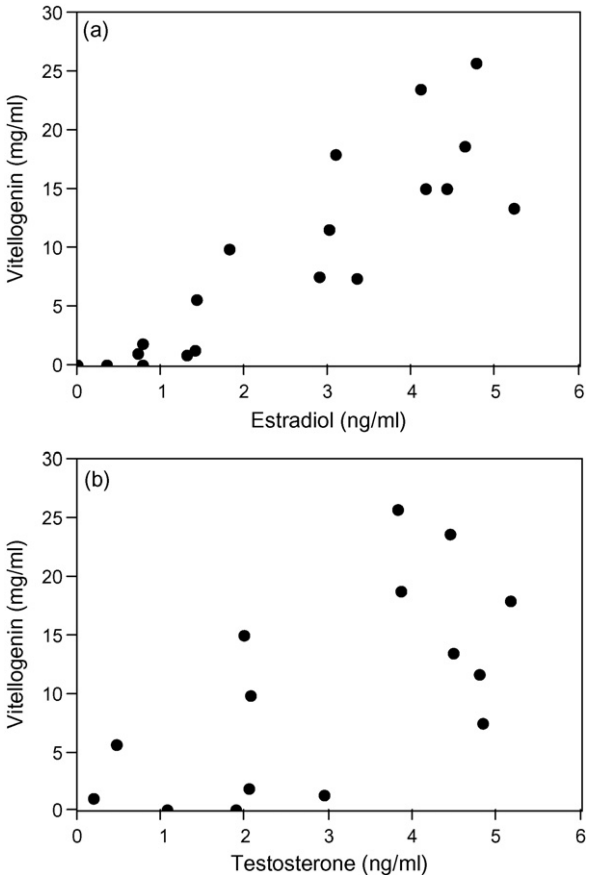

Fig. 2. Relationship between plasma vitellogenin concentrations and plasma concentration of (a) 17β-estradiol, or (b) testosterone in female fathead minnows exposed to fadrozole, prochloraz, 17α- or 17β-trenbolone for 21 d. Fadrozole data are included in panel (a) but not (b). See text for explanation.

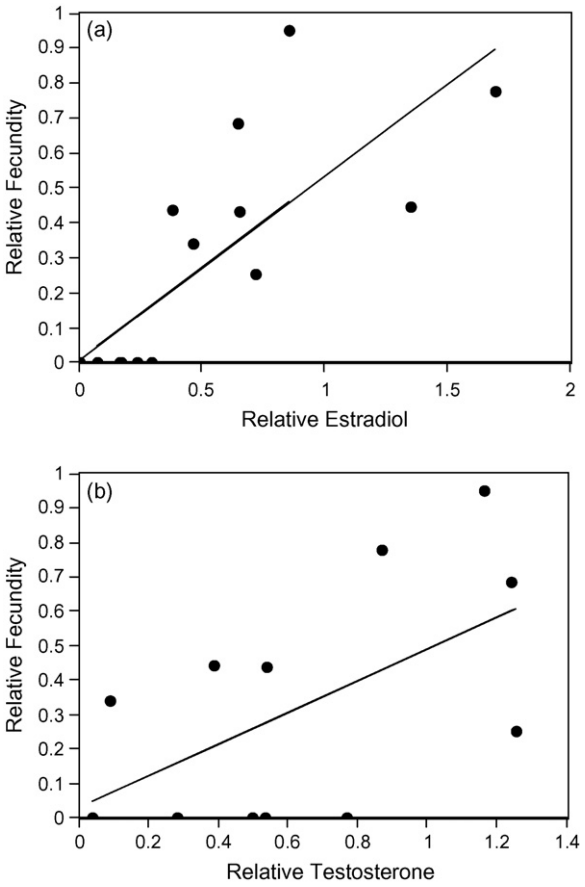

Fig. 3. Relationship between relative fecundity and plasma concentration of (a) 17β-estradiol, or (b) testosterone in female fathead minnows exposed to fadrozole, prochloraz, 17α- or 17β-trenbolone for 21 d. Fadrozole data are included in panel (a) but not (b).

females treated with prochloraz and the two androgen receptor agonists is physiologically consistent (Fig. 1). Given the role of E2 in VTG production, the observed strong correlation ($r = 0.87$) observed between these two variables also is biologically reasonable (Fig. 2a). The correlation between T and VTG was less robust (Fig. 2b; $r = 0.64$) due, perhaps, to a relatively smaller dataset (i.e., three experiments as opposed to four), and because T is one step further removed from VTG production than E2.

There were positive correlations between relative fecundity and both E2 and T ($r = 0.76$ and 0.57, respectively) in female fathead minnows treated with 17α- and β-trenbolone, prochloraz and (in the case of E2) fadrozole (Fig. 3a,b). Regression modeling resulted in significant linear relationships between relative fecundity and plasma concentrations of either of the steroids (Fig. 3a,b):

$$\text{Fecundity} = 0.008 + 0.524\text{E2} \quad (R^2 = 0.58,\ p \leq 0.01) \tag{2}$$

$$\text{Fecundity} = 0.032 + 0.457T \quad (R^2 = 0.32,\ p \leq 0.05) \tag{3}$$

Fig. 4 illustrates population trajectories when Eq. (2) is integrated into the population model. A proportional change in E2 (relative to baseline conditions) was selected, and a corresponding proportional change in fecundity calculated. The Leslie matrix yielded an intrinsic rate of increase equal to 0.337. Where E2 concentrations (and fecundity) of fathead minnows were depressed, the annual fertility rates of the different age classes within the Leslie matrix were adjusted accordingly to account for exposure within population projections. We calculated from Eq. (2) projected trends in population growth for a fathead minnow population at carrying

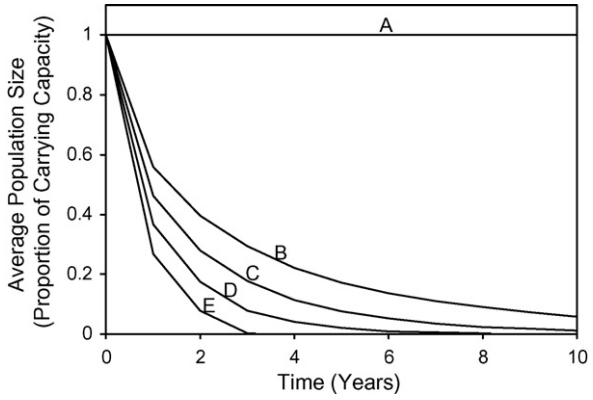

Fig. 4. Relative population trajectories forecasted for a fathead minnow (*Pimephales promelas*) population initially at carrying capacity (baseline conditions) and subsequently exposed to chemicals that depress plasma 17β-estradiol (E2) concentrations. Scenarios illustrated include (A) no reduction in E2, (B) 25% reduction in E2, (C) 50% reduction in E2, (D) 75% reduction in E2, and (E) 100% reduction in E2.

capacity, and subsequently exposed to a chemical stressor(s) that depressed E2 by 25%, 50%, 75%, and 100% (Fig. 4).

Chemical stressors that depress E2 resulted in varying rates of population decline over time, as recorded using an annual time step. As an example, a fathead minnow population existing at carrying capacity exposed to a chemical stressor(s) that depresses E2 by 50% would experience an approximate 92% reduction in population size after 5 years (Fig. 4, line C). The model can also be applied to compare the time frame required to reach a fixed percentage of carrying capacity between different levels of exposure. For instance, exposure to a chemical stressor(s) that depresses E2 levels by 50% and 75% requires only 7 and 4 years, respectively, for the population to decrease to an average size less than 5% of the initial population (Fig. 4, lines C, D). A model was also derived based on the relationship between fecundity and T (Eq. (3)), and similar population-level trends were observed (data not shown).

4. Discussion

We previously showed how VTG concentrations in females could be quantitatively linked to egg production and, through modeling, used to forecast population-level impacts due to inhibition of vitellogenesis (Miller et al., 2007). In the present study we extend that analysis to demonstrate how plasma sex steroid concentrations in females also can be used to make assessments of population trajectories. Specifically, reductions in concentrations of T or E2 in female fathead minnows were shown to be predictive of fecundity of fish exposed to chemicals that affect the HPG axis through inhibition of steroid production. This is a biologically reasonable observation in that VTG production is controlled by E2, the metabolic precursor for which is T. Two of the chemicals used for our analysis, fadrozole and prochloraz, depress steroidogenesis by inhibiting key CYP enzymes in the pathway (Ankley et al., 2002, 2005), while two others, 17α- and β-trenbolone, likely decrease steroid titers through feedback inhibition of the axis (Ankley et al., 2003; Jensen et al., 2006). It is interesting to note that the relationship between VTG and fecundity (Miller et al., 2007) was statistically more robust than that observed between E2 and egg production which, in turn, was more robust that the relationship between T and fecundity. Part of the explanation for this trend almost certainly involves the progressively smaller datasets used to derive the three linear regression models. However, we also speculate that the further removed a particular biochemical/molecular response is from the apical measurement of concern (in this case egg production), the weaker the

predictive relationship may be due to processes such as biological compensation, which could be manifested in a temporally different manner at different points in a pathway.

There is debate as to the appropriate role of biomarkers in ecological risk assessments (Forbes et al., 2006). Most biomarkers employed in ecotoxicology in the past, while sometimes useful as indicators of exposure to chemicals, have been of limited utility relative to prediction of adverse effects. This is due both to the specific biomarkers selected, and the general lack of etiological datasets defining mechanistic linkages between biomarker alterations and progression toward an adverse response(s). The ideal biomarker would be indicative both of the nature (MOA) and apical effects of chemicals. To effectively identify these types of biomarkers it is critical to understand the biological pathways of concern, and the cascade of events whereby chemicals can affect the pathways. The present analysis illustrates how this can be achieved for steroids. Specifically, our data suggest that female sex steroid concentrations (or production) in oviparous animals are reflective both of MOA and reproductive effects. Although steroids control multiple reproductive and developmental processes, production of VTG is one that is essential to egg production. While there undoubtedly are different mechanisms whereby VTG synthesis can be reduced other than through inhibition of steroidogenesis, our current understanding of the function of the HPG axis unequivocally indicates that normal VTG production will not occur without activation of the estrogen receptor. Of course, there will be species-specific differences concerning the degree to which (and when) depressions in steroid production will inhibit egg production, but if the reproductive biology of the species is sufficiently understood, sex steroid status should provide a clear mechanistic linkage to population status.

Numerous studies have evaluated changes in steroid concentrations and/or production as indicative of contaminants that impact the HPG axis. The largest body of work involves fish exposed to pulp and paper mill effluents, which relatively consistently document decreases in steroid production. For example, McMaster et al. (1991) found that adult white sucker exposed to a kraft mill effluent in Jackfish Bay (Ontario, Canada) had depressed plasma concentrations of T (male and female), E2 (female), and KT (male) relative to those observed in fish from reference sites. Similar observations have been made for several other species of fish from different locations affected by pulp mills, suggesting a relatively generalized phenomenon (e.g., Dubé and MacLatchy, 2000, 2001; Karels et al., 2001; Sepúlveda et al., 2001; Fentress et al., 2006). The specific chemical(s) responsible for the effect is uncertain (WHO, 2002), but data suggest an inhibition of gonadal steroid synthesis in exposed animals (Van Der Kraak et al., 1992; McMaster et al., 1995). Alterations in plasma steroid concentrations in fish collected from the field are not limited to those affected by pulp mill effluents (e.g., Goodbred et al., 1997; Nicolas, 1999; Noaksson et al., 2001; Lavado et al., 2004). For example, in a large monitoring study across North America with carp, Goodbred et al. (1997) reported that hormone concentrations varied markedly among various study sites, and that the E2/KT ratio in the fish was associated with concentrations of dissolved pesticides.

Our present analysis provides a conceptual basis for linking depressions in sex steroid concentrations in field-collected (female) fish to possible effects in individuals and populations. However, this is difficult to do in a quantitative fashion with currently available datasets, due in part to cross-species differences in reproductive strategies and, hence, patterns of sex steroid concentrations/production. For example, the fathead minnow is a fractional spawner capable of producing clutches of eggs every 3 d under appropriate conditions (Jensen et al., 2001). Conversely, suckers (for which there is, arguably, the most complete steroid data from the field) are annual spawners, with females typically producing one clutch of eggs in the spring. Nonetheless, it is interesting to consider our fathead minnow analysis in the context of what has been observed in the sucker. For example, the reductions in E2 and T observed by McMaster et al. (1991) in females were on the order of 50–80% (compared to fish from a reference site), well within the range that (based on our fathead minnow model) might be expected to affect fecundity through effects on egg quality. The decreases in steroid concentrations observed by McMaster et al. (1991) were associated with increased age to maturity and decreased gonad size in the female suckers. Interestingly, McMaster et al. (1991) noted that eggs from fish from the effluent-impacted site were lighter and smaller than those from females collected at the reference site, a condition that would be consistent with reduced vitellogenesis.

In addition to lending insights as to the utility of sex steroid status as a biomarker of effects in fish, this analysis is useful in better defining the HPG axis from a systems perspective. We are currently conducting studies focused on developing an organism-level systems biology/toxicology model for reproductive processes controlled by the HPG axis in small fish such as the fathead minnow (Villeneuve et al., 2007). The overall goal of this effort is to translate genomic changes (gene and protein expression, endogenous metabolite profiles) into impacts on reproduction. To achieve this, we are using chemical probes with differing MOA to systematically disrupt the HPG axis, following which responses of the fish are assessed at multiple biological levels of organization. We anticipate that the relationships described in this paper between steroids and fecundity, and by Miller et al. (2007) between VTG and fecundity will help serve as a basis for defining quantitative linkages between genomic responses and apical outcomes in fish exposed to endocrine-active chemicals with different MOA.

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